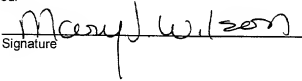


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FORM PTO-1390 (REV. 5/93)		U.S. Department of Commerce Patent and Trademark Office	Attorney's Docket Number 1579-312
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			U.S. Application No. (if known, see 37 C.F.R. 1.5) Unknown
International Application No. PCT/US97/07317	International Filing Date 30 April 1997	Priority Date Claimed 30 April 1996	
Title of Invention METHODS FOR TREATING CANCERS AND PATHOGEN INFECTIONS USING ANTIGEN-PRESENTING CELLS LOADED WITH RNA			
Applicant(s) For DO/EO/US NAIR et al			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.			
<p>1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) Articles 22 and 39(1).</p> <p>4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</p> <p>5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)).</p> <p>a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</p> <p>b. <input type="checkbox"/> has been transmitted by the International Bureau.</p> <p>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</p> <p>7. Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</p> <p>b. <input type="checkbox"/> have been transmitted by the International Bureau</p> <p>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p>d. <input type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (U.S.C. 371(c)(3)).</p> <p>9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p> <p>11. The above checked items are being transmitted:</p> <p>a. <input type="checkbox"/> before the 18th month publication.</p> <p>b. <input type="checkbox"/> after publication and the Article 20 communication but before 20 months from the priority date.</p> <p>c. <input type="checkbox"/> after 20 months.</p> <p>d. <input checked="" type="checkbox"/> by 30 months and a proper demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</p> <p>e. <input type="checkbox"/> after 30 months.</p> <p>Note: Petition to revive (37 CFR 1.137(a) or (b)) is necessary if 35 U.S.C. 371 requirements submitted (1) after 20 months and no proper demand for International Preliminary Examination was made by 19 months from the earliest claimed priority date, or (2) after 30 months and a proper demand for International Preliminary Examination was made by 19 months from the earliest claimed priority date.</p> <p>12. At the time of transmittal, Amendments to the claims under Article 34</p> <p>a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</p> <p>b. <input type="checkbox"/> have been transmitted by the International Bureau</p> <p>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p>d. <input type="checkbox"/> have not been made and will not be made.</p> <p>13. <input type="checkbox"/> Certain requirements under 35 U.S.C. 371 were previously submitted by the applicant on _____, namely:</p> <p>14. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>15. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>16. <input type="checkbox"/> A FIRST preliminary amendment.</p> <p><input type="checkbox"/> A SECOND OR SUBSEQUENT preliminary amendment.</p> <p>17. <input type="checkbox"/> A substitute specification.</p> <p>18. <input type="checkbox"/> A change of power of attorney and/or address letter.</p>			

19. <input checked="" type="checkbox"/> Other items or information: PTO-1449 and International Search Report			
20. <input checked="" type="checkbox"/> The following fees are submitted:		CALCULATION S	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5))			
-- Search Report has been prepared by the EPO or JPO		\$930.00	
-- International preliminary examination fee paid to USPTO (37 CFR 1.492)		\$720.00	
-- No international preliminary examination fee paid to USPTO (37 CFR 1.492) but international search fee paid to USPTO (37 CFR 1.445(a)(2))		\$790.00	
-- Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO		\$1,070.00	
-- International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provision of PCT Article 33(1) to (4)		\$98.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =		\$	720.00
Surcharge of \$130.00 for furnishing the National fee or oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 mos. from the earliest claimed priority date (37 CFR 1.492(e)).		\$	130.00
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total Claims	53	-20 = 33	X \$22.00
Independent Claims	5	-3 = 2	X \$82.00
Multiple Dependent Claims(s) (if applicable)			+ \$270.00
TOTAL OF ABOVE CALCULATIONS =		\$	1740.00
Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed also. (Note 37 CFR 1.9, 1.27, 1.28).			0.00
SUBTOTAL =		\$	1740.00
Processing fee of \$130.00, for furnishing the English Translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 mos. from the earliest claimed priority date (37 CFR 1.492(f)).			0.00
TOTAL NATIONAL FEE =		\$	1740.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +		\$	0.00
Fee for Petition to Revive Unintentionally Abandoned Application (\$1,320 - Small Entity Fee = \$660)		\$	0.00
TOTAL FEES ENCLOSED =		\$	1740.00
		Amount to be refunded	\$
		Charged	\$
a. <input checked="" type="checkbox"/> A check in the amount of \$1740.00 to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. 14-1140 in the amount of \$_____ to cover the above fees. A duplicate copy of this form is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed.			
SEND ALL CORRESPONDENCE TO:		 Signature	
NIXON & VANDERHYE P.C. 1100 North Glebe Road, 8th Floor Arlington, Virginia 22201 Telephone: (703) 816-4000		Mary J. Wilson Name	
32,955		October 29, 1998	
Registration Number		Date	

Applicant or Patentee: Smita K. Nair, David J. Boczkowski, Eli Gilboa

Attorney's

Serial or Patent No.: 09/171,916Dkt. No.: 1579-312Filed or Issued: October 29, 1998For: **METHODS FOR TREATING CANCERS AND PATHOGEN INFECTIONS USING ANTIGEN-PRESENTING CELLS
LOADED WITH RNA****VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY
STATUS (37 1.9(f) and 1.27 (c)) - NONPROFIT ORGANIZATION**

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF
ORGANIZATION DUKE UNIVERSITY

ADDRESS OF
ORGANIZATION Durham, North Carolina 27708-0083

TYPE OF NONPROFIT ORGANIZATION

- ☒ University or other institution of higher education
- ☐ Tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3))
- ☐ Nonprofit scientific or educational under statute of state of The United States of America
(Name of state _____)
(Citation of statute _____)
- ☐ Would qualify as tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3)) if located in the United States
- ☐ Would qualify as nonprofit scientific or educational under statute of state of The United States of America if located in The United States of America
(Name of state _____)
(Citation of statute _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9 (3) for purposes of paying reduced fees to the United States Patent and Trademark Office regarding the invention described in:

- ☐ the specification filed herewith with title as listed above.
- ☒ application identified above.
- ☐ the patent identified above.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention. If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization having any rights in the invention is listed below:

- ☐ no such person, concern, or organization exists.
- ☐ each such person, concern or organization is listed below.*

*Separate verified statement is requested from each person, concern or organization named immediately above having rights to the invention, averring to his/her/its status as a small entity. (37 CFR 1.27)

I acknowledge the duty to file, in this application of patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Joyce McWherter

TITLE IN ORGANIZATION Patent Administrator

ADDRESS OF PERSON SIGNING Duke University, Box 90083, Durham, NC 27708

SIGNATURE Joyce McWherter DATE 1/20/99

300 Rec'd PCT/P/O 29 OCT 1998

METHODS FOR TREATING CANCERS AND PATHOGEN INFECTIONS
USING ANTIGEN-PRESENTING CELLS LOADED WITH RNA

5 Background of the Invention

This invention relates to methods for treating or preventing tumor formation or pathogen infection in a patient.

Previously-described methods for treating cancers
10 include the use of chemotherapeutics, radiation therapy, and selective surgery. The identification of a few tumor antigens has led to the development of cell-based therapies. These methods rely on first identifying a tumor antigen (i.e., a polypeptide that is expressed
15 preferentially in tumor cells, relative to non-tumor cells). Several human tumor antigens have been isolated from melanoma patients, and identified and characterized (Boon and van der Bruggen, 1996, J. Exp. Med. 183: 725-729). These polypeptide antigens can be loaded onto
20 antigen-presenting cells, and then be administered to patients in a method of immunotherapy (i.e., as a vaccine). Alternatively, the polypeptide-loaded antigen-presenting cells can be used to stimulate CTL proliferation ex vivo. The stimulated CTL are then
25 administered to the patient in a method of adoptive immunotherapy.

A variety of methods have been described for treating infections with intracellular pathogens such as viruses and bacteria. For example, antibiotics are
30 commonly used to treat bacterial infections. Preparations of killed pathogens can also serve as vaccines. In addition, CTL-based therapies have been described for treating such infections.

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Summary of the Invention

It has now been discovered that tumor formation in a patient can be treated or prevented by administering to the patient an antigen-presenting cell(s) that is loaded with antigen encoded in RNA derived from a tumor. For convenience, an RNA-enriched tumor preparation can be used in lieu of purified RNA. The invention thus circumvents the need purify RNA or isolate and identify a tumor antigen. Using similar methods and pathogen-derived RNA, pathogen infection in a patient can be treated or prevented. The RNA-loaded antigen-presenting cells can be used to stimulate CTL proliferation *ex vivo* or *in vivo*. The *ex vivo* expanded CTL can be administered to a patient in a method of adoptive immunotherapy.

Accordingly, the invention features a method for producing an RNA-loaded antigen-presenting cell (APC); the method involves introducing into an APC *in vitro* (i) tumor-derived RNA that includes tumor-specific RNA which encodes a cell-surface tumor antigenic epitope which induces T cell proliferation or (ii) pathogen-derived RNA that includes pathogen-specific RNA which encodes a pathogen antigenic epitope that induces T cell proliferation. Upon introducing RNA into an APC (i.e., "loading" the APC with RNA), the RNA is translated within the APC, and the resulting protein is processed by the MHC class I or class II processing and presentation pathways. Presentation of RNA-encoded peptides begins the chain of events in which the immune system mounts a response to the presented peptides.

Preferably, the APC is a professional APC such as a dendritic cell or a macrophage. Alternatively, any APC can be used. For example, endothelial cells and artificially generated APC can be used. The RNA that is loaded onto the APC can be provided to the APC as purified RNA, or as a fractionated preparation of a tumor

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or pathogen. The RNA can include poly A⁺ RNA, which can be isolated by using conventional methods (e.g., use of poly dT chromatography). Both cytoplasmic and nuclear RNA are useful in the invention. Also useful in the invention is RNA encoding defined tumor or pathogen antigens or epitopes, and RNA "minigenes" (i.e., RNA sequences encoding defined epitopes). If desired, tumor-specific or pathogen-specific RNA can be used; such RNA can be prepared using art-known techniques such as subtractive hybridization against RNA from non-tumor cells or against related, but non-pathogenic, bacteria or viruses.

The RNA that is loaded onto APC can be isolated from a cell, or it can be produced by employing conventional molecular biology techniques. For example, RNA can be extracted from tumor cells, reverse transcribed into cDNA, which can be amplified by PCR, and the cDNA then is transcribed into RNA to be used in the invention. If desired, the cDNA can be cloned into a plasmid before it is used as a template for RNA synthesis. RNA that is synthesized *in vitro* can, of course, be synthesized partially or entirely with ribonucleotide analogues or derivatives. Such analogues and derivatives are well known in the art and can be used, for example, to produce nuclease-resistant RNAs. The use of RNA amplification techniques allows one to obtain large amounts of the RNA antigen from a small number of cells.

Included within the invention are methods in which the RNA is isolated from a frozen or fixed tissue. Tumor specimens commonly are isolated from cancer patients and then stored, for example, as cryostat or formalin fixed, paraffin-embedded tissue sections. Because cancer patients often have few tumor cells, the isolation of RNA from fixed tissues is particularly advantageous in

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producing the APCs of the invention because the method can utilize a small tissue sample. Microdissection techniques can be used to separate tumor cells from normal cells. RNA can then be isolated from the tumor cells and amplified *in vitro* (e.g., by PCR or reverse transcription PCR (RT-PCR)). The resulting, amplified RNA then can be used to produce the RNA-loaded APCs described herein.

If desired, RNA encoding an immunomodulator can also be introduced into the APC loaded with tumor-derived or pathogen-derived RNA. In this embodiment, the RNA-encoded immunomodulator is expressed in the APC and enhances the therapeutic effect (e.g., as a vaccines) of the RNA-loaded APCs. Preferably, the immunomodulator is a cytokine or costimulatory factor (e.g., an interleukin, such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, or IL-15, or GM-CSF).

To introduce RNA into an APC, the APC may be contacted with the tumor- or pathogen-derived RNA in the presence of a cationic lipid, such as DOTAP or 1:1 (w/w) DOTMA:DOPE (i.e., LIPOFECTIN). Alternatively, "naked" RNA can be introduced into the cells. Other art-known transfection methods also can be used to introduce the RNA into the APC.

In a variation of the above methods, the RNA that is introduced into the APC can be engineered such that it encodes a cell trafficking signal sequence in addition to a tumor antigen or pathogen antigen. Such an engineered RNA can be thought of as containing two RNA sequences that are covalently linked and which direct expression of a chimeric polypeptide. One RNA sequence encodes the tumor or pathogen antigen, while the other RNA sequence encodes the cell trafficking sequence, thus forming a chimeric polypeptide. The chimeric polypeptides that contain an antigen linked to a trafficking sequence are

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channeled into the MHC class II antigen presentation pathway. Examples of suitable trafficking sequences are provided below.

- Because practicing the invention does not require
- 5 identifying an antigen of the tumor cell or pathogen, RNA derived from essentially any type of tumor or pathogen is useful. For example, the invention is applicable, but not limited, to the development of therapeutics for treating melanomas, bladder cancers, breast cancers,
- 10 pancreatic cancers, prostate cancers, colon cancers, and ovarian cancers. In addition, the invention can treat or prevent infections with pathogens such as *Salmonella*, *Shigella*, *Enterobacter*, human immunodeficiency virus, Herpes virus, influenza virus, poliomyelitis virus,
- 15 measles virus, mumps virus, or rubella virus.

- The antigen-presenting cells produced in accordance with the invention can be used to induce CTL responses *in vivo* and *ex vivo*. Thus, the invention includes methods for treating or preventing tumor
- 20 formation in a patient by administering to the patient a therapeutically effective amount of APC loaded with tumor-derived RNA. The tumor-derived RNA can be derived from the patient, e.g., as an RNA-enriched tumor preparation. Alternatively, the tumor-derived RNA used
- 25 in such a treatment regimen can be derived from another patient afflicted with the same, or a similar, type of cancer. Likewise, APC loaded with pathogen-derived RNA can be used to treat or prevent a pathogen infection in a patient.
- 30 Included within the invention are methods for producing a cytotoxic T lymphocyte. Such a CTL can be produced by contacting a T lymphocyte *in vitro* with an antigen-presenting cell that is loaded with tumor-derived or pathogen-derived RNA, and maintaining the T lymphocyte
- 35 under conditions conducive to CTL proliferation, thereby

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- producing a CTL. The resulting CTL show remarkable specificity for the pathogen or the cells of the tumor from which the loaded RNA is derived. Such CTL can be administered to a patient in a variation of conventional adoptive immunotherapy methods. If desired, one can assay for sensitization (i.e., activation) of the CTL. To this end, any of the art-known assays, such as cytotoxicity assays, can be used. For example, the cytotoxicity assay can include detecting killing of an RNA-loaded cell(s) (e.g., fibroblasts or dendritic cells produced as described herein (i.e., an RNA-loaded cell) that presents on its surface a tumor antigenic epitope or pathogen antigenic epitope encoded by the RNA). Another suitable method for detecting CTL sensitization is by detecting an increase in cytokine secretion, relative to the level of cytokine secretion (e.g., TNF- α or γ -interferon) obtained prior to contacting the CTL (e.g., in an *in situ* immunoassay, such as an ELISPOT assay).

- In a variation of the above methods, the invention provides a method for generating a tumor-specific (or pathogen-specific) CTL response. Because most cancer patients naturally display a non-detectable or poor tumor-specific CTL response, this method is particularly useful since it provides a method for producing a CTL response using antigens obtained from any patient. Once a CTL response is generated, one can use conventional methods to identify the antigens that induce the CTL response. For example, antigens in tumor extracts can be fractionated (e.g., by HPLC), and the fractions can be assayed to determine which fraction contains an antigen that is recognized by the tumor-specific CTL produced in accordance with the invention. The method of the invention thus serves as a platform for antigen discovery. The method entails:

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a) introducing into an antigen-presenting cell *in vitro* polyA⁺ RNA that includes at least 80% (preferably at least 90% or 100%) of the polyA⁺ RNA species, thereby producing an RNA-loaded APC; and

5 b) contacting a T lymphocyte with the RNA-loaded APC, thereby producing a tumor-specific or pathogen-specific CTL response. If desired, induction of such a CTL response can be detected by detecting sensitization of the contacted T lymphocyte using the methods described
10 herein. In practicing this method, unfractionated, total tumor-derived (or pathogen-derived) RNA typically will be used to produce the RNA-loaded APC, since total RNA is certain to contain an RNA species that encodes the tumor antigen (or pathogen antigen).

15 The invention also includes methods for treating or preventing tumor formation in a patient by administering to the patient a therapeutically effective amount of APC loaded with tumor-derived RNA. Similarly, the invention provides methods for treating pathogen
20 infection in a patient by administering to the patient a therapeutically effective amount of APC loaded with pathogen-derived RNA. The T lymphocytes that are used in these various therapeutic methods can be derived from the patient to be treated, or haplotype-matched CTL from a
25 donor can be used. Similarly, the RNA used in these methods can be derived from the patient to be treated, or RNA from a donor can be used.

By "RNA-loaded" or "RNA-pulsed" antigen-presenting cell is meant an APC (e.g., a macrophage or dendritic
30 cell) that was incubated or transfected with RNA, e.g., RNA derived from a tumor or pathogen. Such RNA can be loaded onto the APC by using conventional nucleic acid transfection methods, such as lipid-mediated transfection, electroporation, and calcium phosphate
35 transfection. For example, RNA can be introduced into

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APC by incubating the APC with the RNA (or extract) for 1 to 24 hours (e.g., 2 hours) at 37°C, preferably in the presence of a cationic lipid.

- By "tumor-derived" RNA is meant a sample of RNA that has its origin in a tumor cell, and which includes RNA corresponding to a tumor antigen(s). Included is RNA that encodes all or a portion of a previously identified tumor antigen. Similarly "pathogen-derived" RNA is a sample of RNA that has its origin in an pathogen (e.g., a bacterium or virus, including intracellular pathogens). Such RNA can be "in vitro transcribed," e.g., reverse transcribed to produce cDNA that can be amplified by PCR and subsequently be transcribed in vitro, with or without cloning the cDNA. Also included is RNA that is provided as a fractionated preparation of tumor cell or pathogen. Because even unfractionated RNA preparation (e.g., total RNA or total poly A+ RNA) can be used, it is not necessary that a tumor or pathogen antigen be identified. In one embodiment, the preparation is fractionated with respect to a non-RNA component(s) of the cell in order to decrease the concentration of a non-RNA component, such as protein, lipid, and/or DNA and enrich the preparation for RNA. If desired, the preparation can be further fractionated with respect to the RNA (e.g., by subtractive hybridization) such that "tumor-specific" or "pathogen-specific" RNA is produced.

- By "tumor-specific" RNA is meant an RNA sample that, relative to unfractionated tumor-derived RNA, has a high content of RNA that is preferentially present in a tumor cell compared with a non-tumor cell. For example, tumor-specific RNA includes RNA that is present in a tumor cell, but not present in a non-tumor cell. Also encompassed in this definition is an RNA sample that includes RNA that is present both in tumor and non-tumor cells, but is present at a higher level in tumor cells

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- than in non-tumor cells. Also included within this definition is RNA that encodes a previously identified tumor antigen and which is produced *in vitro*, e.g., from a plasmid or by PCR. Alternatively, tumor-specific RNA
- 5 can be prepared by fractionating an RNA sample such that the percentage of RNA corresponding to a tumor antigen is increased, relative to unfractionated tumor-derived RNA. For example, tumor-specific RNA can be prepared by fractionating tumor-derived RNA using conventional
- 10 subtractive hybridization techniques against RNA from non-tumor cells. Likewise, "pathogen-specific" RNA refers to an RNA sample that, relative to unfractionated pathogen-derived RNA, has a high content of RNA that is preferentially present in the pathogen compared with a
- 15 non-pathogenic strain of bacteria or virus.

- By "trafficking sequence" is meant an amino acid sequence (or an RNA encoding an amino acid sequence) that functions to control intracellular trafficking (e.g., directed movement from organelle to organelle or to the
- 20 cell surface) of a polypeptide to which it is attached.

- The invention offers several advantages. Vaccinations performed in accordance with the invention circumvent the need to identify specific tumor rejection antigens or pathogen antigens, because the correct
- 25 antigen(s) is automatically selected from the tumor- or pathogen-derived RNA when unfractionated RNA is used. If desired, the risk of generating an autoimmune response can be diminished by using tumor-specific RNA. In addition, vaccination with cells loaded with
- 30 unfractionated tumor-derived RNA likely elicits immune responses to several tumor antigens, reducing the likelihood of "escape mutants." The invention also extends the use of active immunotherapy to treating cancers for which specific tumor antigens have not yet
- 35 been identified, which is the vast majority of cancers.

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Furthermore, the RNA to be introduced into APCs can be derived from fixed tissue samples. Fixed samples of tumor tissues are routinely prepared in the course of diagnosing cancer; thus, the use of RNA from such samples does not require subjecting a patient to an additional invasive procedure. Because most cancer patients have low tumor burdens, the methods of the invention that involve isolation and amplification of RNA from fixed tumor tissues are particularly valuable. The invention can be used efficaciously even if the tumor itself displays poor immunogenicity. In addition, the invention is useful for reducing the size of preexisting tumors, including metastases even after removal of the primary tumor. Finally, the invention offers the advantage that antigen-presenting cells that are loaded with *in vitro* transcribed RNA can be more potent vaccines than are antigen-presenting cells that are loaded with peptide antigens.

Brief Description of the Drawings

Fig. 1 is a graph illustrating primary OVA-specific CTL induction *in vitro* with dendritic cells pulsed with RNA. DC were pulsed with total RNA or poly A⁺ RNA obtained from E.G7-OVA or 'EL4 cells, or *in vitro* transcribed OVA RNA in the presence of the cationic lipid DOTAP as described herein. DC pulsed with the OVA peptide were used for comparison. DC and naive T cells were incubated for 5 days at a R/S of 20:1. Viable lymphocytes were harvested, and the CTL activity was determined in a routine europium release assay. E.G7-OVA and EL4 cells were used as targets. This experiment was repeated three times with similar results.

Fig. 2 is a graph illustrating that the sensitization of E.G7-OVA RNA pulsed DC for stimulation of OVA-specific primary CTL responses is mediated by the

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poly A⁺ fraction of RNA. DC were pulsed with total RNA, poly A⁻ RNA or poly A⁺ RNA, and cultured with naive T cells in 96-well U-bottom plates for 5 days. The poly A⁺ RNA fraction from E.G7-OVA cells was treated with an
5 antisense oligonucleotide specific for the CTL epitope encoding region of the OVA gene, or a control oligonucleotide followed by RNase H treatment to eliminate the hybridized RNA. DC pulsed with OVA peptide was used as a control. E.G7-OVA, EL4, and RMA cells
10 pulsed with the OVA peptide were used as targets.

Fig. 3 is a histogram depicting the induction of anti-tumor immunity in vivo in mice following a single immunization with DC pulsed with RNA. DC were pulsed with either total or poly A⁺ RNA from E.G7-OVA cells or
15 EL4 cells, or with *in vitro* transcribed OVA RNA or control antisense OVA RNA. Mice were immunized with 2 x 10⁶ DC or 5 x 10⁶ irradiated E.G7-OVA or EL4 cells injected intraperitoneally, followed by a challenge with 2 x 10⁷ live E.G7-OVA cells. Mice were periodically
20 examined for tumor growth, and were sacrificed when the tumor diameter reached 3-4 cm. All mice were sacrificed at 35-40 days post-challenge.

Fig. 4 is a histogram depicting the regression of spontaneous metastasis in mice vaccinated with DC pulsed
25 with poly A⁺ RNA or total RNA in the B16-F10.9 melanoma model. Mice received by intrafootpad injection live F10.9 cells, and the legs were amputated when the tumor diameter reached 5.5-7.5 mm. Vaccinations were initiated 2 days post-amputation, and were followed by two more
30 vaccinations at weekly intervals. Mice were vaccinated intraperitoneally with 2 x 10⁶ total, poly A⁻ or poly A⁺ RNA pulsed DC, or irradiated F10.9 cells or F10.9/K1 cells, or PBS (as a control.) Mice were sacrificed based on the metastatic death in the non-immunized or control
35 groups (28-32 days post-amputation). Metastatic loads

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were assayed by weighing the lungs and by counting the number of metastatic nodules.

Detailed Description

Before providing detailed working examples of the invention, certain parameters of the invention will be described generally.

A variety of methods are suitable for producing the tumor- or pathogen-derived RNA that can be used in the invention. As the following examples illustrate, it is not necessary that the RNA be provided to the APC in a purified form. Preferably, the RNA sample (i.e., the fractionated tumor preparation or IVT RNA sample) is at least 50%, more preferably 75%, 90%, or even 99% RNA (wt/vol). In practicing the invention, antigen-presenting cells, preferably professional APC such as dendritic cells and macrophage, are used. Such cells can be isolated according to previously-described procedures.

Any of a variety of methods can be used to produce RNA-containing tumor preparations. For example, the tumor preparations can be produced by sonicating tumor cells in a mammalian cell culture medium such as Opti-MEM or a buffer such as phosphate buffered saline. Similarly, pathogen-derived RNA can be produced by sonicating pathogenic bacteria or cells containing a pathogenic virus. Other methods for disrupting cells also are suitable, provided that the method does not completely degrade the tumor- or pathogen-derived RNA. Typically, the RNA preparation has 10^6 to 10^8 cells/ml; most preferably 10^7 cells/ml. As alternatives, or in addition, to sonication, the tumor- or pathogen-derived RNA can be prepared by employing conventional RNA purification methods such as guanidinium isothiocyanate methods and/or oligo dT chromatography methods for isolating poly A⁺ RNA. IVT RNA, synthesized according to

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conventional methods, can be used in lieu of RNA in tumor preparations. For example, RNA from a tumor or pathogen can be reverse transcribed into cDNA, which then is amplified by conventional PCR techniques to provide an essentially unlimited supply of cDNA corresponding to the tumor or pathogen RNA antigen. Conventional *in vitro* transcription techniques and bacterial polymerases then are used to produce the IVT RNA. As an alternative, the IVT RNA can be synthesized from a cloned DNA sequence encoding a tumor or pathogen polypeptide antigen. Methods for identifying such antigens are known in the art; for example, several melanoma peptide antigens have been identified. RNA transcribed *in vitro* from cDNA encoding identified peptide antigens can serve as tumor- or pathogen-specific RNA in the invention. As an alternative, RNA can be transcribed from "minigenes" consisting of a portion of the tumor antigen cDNA that encodes an epitope. Tumor- or pathogen-specific RNA can also be produced by employing conventional techniques for subtractive hybridization. For example, an RNA sample from tumor cells and non-tumor cells can be used in the subtractive hybridization method to obtain tumor-specific RNA.

If desired, the tumor-derived or pathogen-derived RNA can be prepared from frozen or fixed tissues. Although not required, the tissue sample can be enriched for tumor-specific RNA. Microdissection techniques that are suitable for separating tumor cells from non-tumor cells have been described (Zhuang et al., 1995, Cancer Res. 55:467-471; Luqmani et al., 1992, Anal. Biochem. 200:291-295; Luqmani et al., 1994, Anal. Biochem. 222:102-109; Turbett et al., 1996, BioTech. 20:846-853). Once the tumor cells are separated from the non-tumor cells, tumor-derived RNA can be isolated from the tumor cells using art-known techniques. For example, tumor-

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derived poly-A⁺ RNA can be isolated by hybridizing the RNA to a solid phase, such as oligo(dT) linked to paramagnetic beads (see, e.g., Raineri et al., 1991, Nucl. Acids. Res. 19:4010). The first cDNA strand then is synthesized by reverse transcription, the RNA is removed, and the second strand is synthesized (e.g., with DNA polymerase). Conventional *in vitro* transcription methods then can be used to synthesize the RNA. Other art-known methods for amplifying RNA from a small number of cells, or even a single cell, also can be used in the invention.

An RNA molecule that encodes a tumor or pathogen antigenic epitope can, if desired, be engineered such that it also encodes a cell trafficking signal sequence.

Such a chimeric RNA molecule can be produced using conventional molecular biology techniques. The chimeric RNA that is introduced into an APC encodes a chimeric polypeptide, which contains an antigen linked to a trafficking sequence that directs the chimeric polypeptide into the MHC class II antigen presentation pathway. For example, the trafficking sequences employed in this embodiment of the invention may direct trafficking of the polypeptide to the endoplasmic reticulum (ER), a lysosome, or an endosome, and include signal peptides (the amino terminal sequences that direct proteins into the ER during translation), ER retention peptides such as KDEL (SEQ ID NO: 1); and lysosome-targeting peptides such as KFERQ (SEQ ID NO: 2), QREK (SEQ ID NO: 3), and other pentapeptides having Q flanked on one side by four residues selected from K, R, D, E, F, I, V, and L. A preferred signal peptide that can be used in the invention is the LAMP-1 sorting signal (Wu et al., 1995, Proc. Natl. Acad. Sci. 92:11671-11675; Lin et al., 1996, Cancer Research 56: 21-26). Another example of a signal peptide that is useful in the invention is a

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signal peptide substantially identical to that of an MHC subunit such as class II α or β ; e.g., the signal peptide of MHC class II α is contained in the sequence MAISGVPLGFFIIAVLMSAQESWA (SEQ ID NO: 4). If desired,

- 5 the signal peptide encoded by the RNA of the invention may include only a portion (typically at least ten amino acid residues) of the specified 25 residue sequence, provided that portion causes trafficking of the polypeptide to the ER.

- 10 Transfection methods that are suitable for introducing the tumor- or pathogen-derived RNA into an antigen-presenting cell are known in the art. For example, 5-50 μg of RNA in 500 μl of Opti-MEM can be mixed with a cationic lipid at a concentration of 10 to 15 100 μg , and incubated at room temperature for 20 to 30 minutes. Other suitable lipids include LIPOFECTIN[™] (1:1 (w/w) DOTMA:DOPE), LIPOFECTAMINE[™] (3:1 (w/w) DOSPA:DOPE), DODAC:DOPE (1:1), CHOL:DOPE (1:1), DMEDA, CHOL, DDAB, DMEDA, DODAC, DOPE, DORI, DORIE, DOSPA, DOTAP, and DOTMA.
- 20 The resulting RNA-lipid complex is then added to 1-3 x 10⁶ cells, preferably 2 x 10⁶, antigen-presenting cells in a total volume of approximately 2 ml (e.g., in Opti-MEM), and incubated at 37° C for 2 to 4 hours. Alternatively, the RNA can be introduced into the antigen presenting 25 cells by employing conventional techniques, such as electroporation or calcium phosphate transfection with 1-5 x 10⁶ cells and 5 to 50 μg of RNA. Typically, 5-20 μg of poly A⁺ RNA or 25-50 μg of total RNA is used.

- When the RNA is provided as a tumor or pathogen 30 preparation, the preparation typically is fractionated or otherwise treated to decrease the concentration of proteins, lipids, and/or DNA in the preparation, and enrich the preparation for RNA. For example, art-known RNA purification methods can be used to at least 35 partially purify the RNA from the tumor cell or pathogen.

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It is also acceptable to treat the RNA preparation with proteases or RNase-free DNases. Of course, the RNA can be synthesized using art-known nuclease-resistant analogues or derivatives in order to render the RNA less susceptible to ribonucleases.

If desired, RNA encoding an immunomodulator, such as a cytokine or a co-stimulatory factor, can be introduced into the RNA-loaded APCs of the invention. In this embodiment of the invention, the RNA encoding the immunomodulator may be introduced into the APC prior to, simultaneously with, or subsequent to introduction of the tumor- or pathogen-derived RNA. The methods described herein for introducing the tumor-derived or pathogen-derived RNA into the APC also are suitable for introducing into the APC RNA encoding an immunomodulator (e.g., a cytokine or costimulatory factor). Sequences encoding numerous proteins are known in the art and can be used in the invention. If desired, RNA encoding two or more immunomodulators can be introduced into the APC. Typically, 5-20 μ g of each RNA is introduced into the APC, as is described above for tumor- and pathogen-derived RNA.

The RNA-loaded antigen-presenting cells of the invention can be used to stimulate CTL proliferation *in vivo* or *ex vivo*. The ability of RNA-loaded antigen-presenting cells to stimulate a CTL response can be measured or detected by measuring or detecting T-cell activation, for example, in a conventional cytotoxicity assay. In examples provided below, the cytotoxicity assay entails assaying the ability of the effector cells to lyse target cells. If desired, the target cells can be RNA-loaded APCs produced in accordance with the invention (i.e., APCs that present an RNA-encoded cell-surface tumor or pathogen antigenic epitope that induces T cell proliferation). As is described below, the

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commonly-used europium release assay can be used to assay CTL sensitization. Typically, $5-10 \times 10^6$ target cells are labeled with europium diethylenetriamine pentaacetate for 20 minutes at 4°C. After several washes 10^4

- 5 europium-labeled target cells and serial dilutions of effector cells at an effector:target ratio ranging from 50:1 to 6.25:1 are incubated in 200 μ l RPMI 1640 with 10% heat-inactivated fetal calf serum in 96-well plates. The plates are centrifuged at $500 \times g$ for 3 minutes and the
- 10 incubated at 37° C in 5% CO₂ for 4 hours. A 50 μ l aliquot of the supernatant is collected, and europium release is measured by time resolved fluorescence (Volgmann et al., J. Immunol. Methods 119:45-51, 1989).

- In an alternative method for detecting CTL
- 15 sensitization, an increase in cytokine secretion by the CTL is detected, relative to the level of cytokine secretion prior to contacting the CTL with an RNA-loaded APC. *In situ* hybridization assays, such as ELISPOT assays, can be used to detect secretion of cytokines such
- 20 as TNF- α and/or γ -interferon.

Examples

The following working examples are meant to illustrate, not limit, the invention. First, the methods used in these examples are described.

25 Mice

Seven to eight weeks old and retired breeder female C57BL/6 mice (H-2^b) were obtained from the Jackson Laboratory (Bar Harbor, ME).

Cell lines

- 30 The F10.9 clone of the B16 melanoma of C57BL/6 origin is a highly metastatic, poorly immunogenic, and low class I expressing cell line. F10.9/K1 is a poorly

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metastatic and highly immunogenic cell line derived by transfecting F10.9 cells with class I molecule, H-2K^b cDNA. RMA and RMA-S cells are derived from the Rauscher leukemia virus-induced T cell lymphoma RBL-5 of C57BL/6 (H-2^b) origin. Other cell lines used were EL4 (C57BL/6, H-2^b, thymoma), E.G7-OVA (EL4 cells transfected with the cDNA of chicken ovalbumin (OVA), A20(H-2^d B cell lymphoma) and L929 (H-2^k fibroblasts). Cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 25 mM Hepes, 2 mM L-glutamine and 1 mM sodium pyruvate. E.G7-OVA cells were maintained in medium supplemented with 400 µg/ml G418 (GIBCO, Grand Island, NY) and F10.9/K1 cells were maintained in medium containing 800 µg/ml G418.

15 Antigen presenting cells and responder T cells

Splenocytes obtained from naive C57BL/6 female retired breeders were treated with ammonium chloride Tris buffer for 3 minutes at 37°C to deplete red blood cells. Splenocytes (3 ml) at 2×10^7 cells/ml were layered over a 2 ml metrizamide gradient column (Nycomed Pharma AS, Oslo, Norway; analytical grade, 14.5 g added to 100 ml PBS, pH 7.0) and centrifuged at 600 g for 10 minutes. The dendritic cell-enriched fraction from the interface was further enriched by adherence for 90 minutes.

25 Adherent cells (mostly dendritic cells (DC) and a few contaminating macrophage (Mø) were retrieved by gentle scraping, and subjected to a second round of adherence at 37°C for 90 minutes to deplete the contaminating Mø. Non-adherent cells were pooled as splenic DC and FACS

30 analysis showed approximately 80% - 85% DC (mAb 33D1), 1-2% Mø (mAb F4/80), 10% T cells, and <5% B Cells (data not shown).

The pellet was resuspended and enriched for Mø by two rounds of adherence at 37°C for 90 minutes each.

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More than 80% of the adherent population was identified as Mø by FACS analysis, with 5% lymphocytes and <55% DC.

B cells were separated from the non-adherent population (B and T cells) by panning on anti-Ig coated plates. The separated cell population, which was comprised of >80% T lymphocytes by FACS analysis was used as responder T cells.

Isolation of total and poly A⁺ cellular RNA

Total RNA was isolated from actively growing tissue culture cells as previously described (Chomczynski and Sacchi, 1987, Anal. Biochem. 162: 156-159). Briefly, 10⁷ cells were lysed in 1 ml of guanidinium isothiocyanate (GT) buffer (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7.0; 0.5% sarcosyl, 20 mM EDTA, and 0.1 M 2-mercaptoethanol). Samples were vortexed, and followed by sequential addition of 100 µl 3 M sodium acetate, 1 ml water-saturated phenol and 200 µl chloroform:isoamyl alcohol (49:1). Suspensions were vortexed and then placed on ice for 15 minutes. The tubes were centrifuged at 10000 x g, at 4°C for 20 minutes, and the supernatant was carefully transferred to a fresh tube. An equal volume of isopropanol was added and the samples were placed at -20°C for at least 1 hour. RNA was pelleted by centrifugation as above. The pellet was resuspended in 300 µl GT buffer, and then transferred to a microcentrifuge tube. RNA was again precipitated by adding an equal volume of isopropanol and placing the tube at -20°C for at least 1 hour. Tubes were microcentrifuged at high speed at 4°C for 20 minutes. Supernatants were decanted, and the pellets were washed once with 70% ethanol. The pellets were allowed to dry at room temperature and then resuspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). Possible contaminating DNA

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was removed by incubating the RNA sample in 10 mM MgCl₂, 1 mM DTT and 5 U/ml RNase-free DNase (Boehringer-Mannheim) for 15 minutes at 37°C. The solution was adjusted to 10 mM Tris, 10 mM EDTA, 0.5% SDS and 1 mg/ml Pronase (Boehringer-Mannheim), followed by incubation at 37°C for 30 minutes. Samples were extracted once with phenol-chloroform and once with chloroform; RNA was again precipitated in isopropanol at -20°C. Following centrifugation, the pellets were washed with 70% ethanol, then air dried and resuspended in sterile water. Total RNA was quantitated by measuring the optical density (OD) at 260 and 280 nm. The OD 260/280 ratios were typically 1.65-2.0. The RNA was stored at -70°C.

Poly A⁺ RNA was isolated either from total RNA using an OLIGOTEX[™] poly A⁺ purification kit (Qiagen), or directly from tissue culture cells using the Messenger RNA Isolation kit (Stratagene) as per the manufacturer's protocols. If desired, alternative, conventional methods can be used to prepare poly A⁺ RNA.

20 Production of in vitro transcribed RNA

The 1.9 kb EcoRI fragment of chicken ovalbumin cDNA in pUC18 (McReynolds et al., 1978, Nature 273:723) containing the coding region and 3' untranslated region, was cloned into the EcoRI site of pGEM4Z (Promega).

Clones containing the insert in both the sense and anti-sense orientations were isolated, and large scale plasmid preps were made using Maxi Prep Kits[™] plasmid preparation kit (Qiagen). Plasmids were linearized with BamHI for use as templates for *in vitro* transcription.

Transcription was carried out at 37°C for 3-4 hours using the MEGAscript In Vitro Transcription Kit[™] (Ambion) according to the manufacturer's protocol and adjusting the GTP concentration to 1.5 mM and including 6 mM m⁷G(5¹)ppp(5¹)G cap analog (Ambion). Other, conventional

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in vitro transcription methods also are suitable.

Template DNA was digested with RNase-free DNase 1, and RNA was recovered by phenol:chloroform and chloroform extraction, followed by isopropanol precipitation. RNA was pelleted by microcentrifugation, and the pellet was washed once with 70% ethanol. The pellet was air-dried and resuspended in sterile water.

RNA was incubated for 30 minutes at 30°C in 20 mM Tris-HCl, pH 7.0, 50 mM KCl, 0.7 mM MnCl₂, 0.2 mM EDTA, 100 µg/ml acetylated BSA, 10% glycerol, 1 mM ATP and 5000 U/ml yeast poly (A) polymerase (United States Biochemical). The capped, polyadenylated RNA was recovered by phenol:chloroform and chloroform extraction followed by isopropanol precipitation. RNA was pelleted by microcentrifugation, and the pellet was washed once with 70% ethanol. The pellet was air-dried and resuspended in sterile water. RNA was quantitated by measuring the OD at 260 and 280 nm, and the RNA stored at -70°C.

20 Oligodeoxynucleotide directed cleavage of OVA mRNA by RNase H

The procedure used for RNase H site-specific cleavage of ovalbumin mRNA was adapted from those previously described (Donis-Keller, 1979, Nucl. Acid.

25 Res. 7: 179-192). Briefly, 5-10 µg mRNA from E.G7-OVA cells was suspended in 20 mM HEPES-KOH, pH 8.0, 50 mM KCl, 4 mM MgCl₂, 1 mM DTT, 50 µg/ml BSA and 2 µM of either the oligodeoxynucleotide 5' -CAG TTT TTC AAA GTT GAT TAT ACT-3' (SEQ ID NO: 5) , 30 which hybridizes to sequence in OVA mRNA that codes for the CTL epitope SIINFEKL (SEQ ID NO: 6), or 5'-TCA TAT TAG TTG AAA CTT TTT GAC-3' (SEQ ID NO: 7) (Oligos, Etc.), which serves as a negative control. The samples were heated to 50°C for 3 minutes followed by incubation at

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- 37°C for 30 minutes. RNase H (Boehringer-Mannheim) was added at 10 U/ml, and digestion proceeded for 30 minutes at 37°C. RNA was recovered by phenol:chloroform and chloroform extraction, followed by isopropanol
- 5 precipitation. RNA was pelleted by microcentrifugation, and the pellet was washed once with 70% ethanol. The pellet then was air-dried and resuspended in sterile water. Cleavage of OVA mRNA was confirmed by oligo dT primed reverse transcription of test and control samples,
- 10 followed by PCR with OVA specific primers that flank the cleavage site. PCR with actin-specific primers was used to control between test and control samples.

Pulsing of APC

- APC were washed twice in Opti-MEM medium (GIBCO,
- 15 Grand Island, NY). Cells were resuspended in Opti-MEM medium at $2-5 \times 10^6$ cells/ml, and added to 15 ml polypropylene tubes (Falcon). The cationic lipid DOTAP (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used to deliver RNA into cells (Walker et al., 1992,
- 20 Proc. Natl. Acad. Sci. USA 89: 7915-7918). RNA (in 250-500 μ l Opti-MEM medium) and DOTAP (in 250-500 μ l Opti-MEM medium) was mixed in a 12 x 75 mm polystyrene tube at room temperature (RT) for 20 minutes. The RNA to DOTAP ratio routinely used was 1:2, and varied in certain
- 25 experiments between 2:1 to 1:2. The complex was added to the APC ($2-5 \times 10^6$ cells) in a total volume of 2 ml and incubated at 37°C in a water-bath with occasional agitation for 2 hours. The cells were washed and used as stimulators for primary CTL induction *in vitro*.
- 30 The synthetic peptide encoding the CTL epitope in chicken ovalbumin OVA, aa 257-264 SIINFEKL (H-2K^b) (SEQ ID NO: 6), was used for peptide pulsing. The peptide had unblocked (free) amino and carboxyl ends (Research

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Genetics, Birmingham, AL). Peptides were dissolved in serum-free IMDM and stored at -20°C.

Induction of CTL *in vitro*

- 5 T cells (5×10^6 cells/ml) and RNA or peptide pulsed APC (2.5×10^5 cells/ml) were cultured in IMDM with 10% FCS, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 5×10^{-5} M β -mercaptoethanol in 96 well U-bottom plates to give an R/S ratio of 20:1.
- 10 After 5 days, cells were used as effectors in a standard 4 hours europium release assay.

Cytotoxicity assay

- In these assays, $5-10 \times 10^6$ target cells were labeled with europium diethylenetriamine pentaacetate for
- 15 20 minutes at 4°. After several washes, 10^4 europium-labeled targets and serial dilutions of effector cells at effector:target ratios of 50:1 to 6.25:1 were incubated in 200 μ l of RPMI 1640 with 10% heat-inactivated FCS in 96-well V-bottom plates. The plates were centrifuged at
- 20 500 g for 3 minutes and incubated at 37°C and 5% CO₂ for 4 hours. 50 μ l of the supernatant was harvested, and europium release was measured by time resolved fluorescence (Delta fluorometer, Wallace Inc., Gaithersburg, MD). Spontaneous release was less than
- 25 25%. Standard errors (SE) of the means of triplicate cultures was less than 5%.

Immunotherapy

- E.G7-OVA model: C57BL/6 mice were immunized once with irradiated, RNA-pulsed APC (2×10^6 cells/mouse) or
- 30 5×10^6 E.G7-OVA or EL4 cells. At 10-14 days post-immunization, mice were challenged with 2×10^7 live E.G7-OVA cells injected sub-cutaneously in the flank

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region. Mice were monitored on a regular basis for tumor growth and size. Mice with tumor sizes >3.5 cm were sacrificed. All survivors were sacrificed at 40 days post-challenge.

- 5 F10.9-B16 melanoma model: Mice were received by intrafootpad injection 2×10^5 F10.9 cells. The post-surgical protocol was essentially as described previously (Porgador et al., 1995, Cancer Res. 55: 4941-4949). The legs of the mice were amputated when the local tumor in
- 10 the footpad was 5.5 - 7.5 mm in diameter. Post-amputation mortality was less than 5%. At two days post-amputation, the mice were immunized intraperitoneally, followed by weekly vaccinations twice, for a total of three vaccinations. The mice were sacrificed based on
- 15 the metastatic death in the non-immunized or control groups (at 28-32 days post-amputation). The metastatic loads were assayed by weighing the lungs and by counting the number of metastatic nodules.

Induction of a primary CTL response in vitro using

- 20 dendritic cells transfected with chicken ovalbumin RNA.

The ability of RNA pulsed splenic dendritic cells (DC) derived from C57BL/6 (H-2K^b) mice to induce a primary CTL response in vitro was demonstrated in the E.G7-OVA tumor system. E.G7-OVA cells were derived from

25 the EL4 tumor cell line (H-2K^b haplotype) by transfection with the chicken ovalbumin cDNA (Moore et al., 1988, Cell 54: 777-785). The chicken ovalbumin encodes a single dominant epitope (aa 257-264) in C57BL/6 mice (Rotzschke et al., 1991, Euro. Journal Immunology, 21: 2891-2891).

- 30 Dendritic cells pulsed with the OVA peptide (aa 257-264) incubated with T cells from naive mice induce a potent CTL response in vitro (Fig. 1). This example demonstrates that RNA can be used as a source of antigen to sensitize DC to present antigen to CD8⁺ T cells.

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- Splenic DC were isolated from C57BL/6 mice and pulsed with OVA peptide or incubated with RNA synthesized *in vitro* (OVA IVT RNA) from a plasmid encoding the chicken ovalbumin cDNA, and used to stimulate an OVA-specific primary CTL response *in vitro*. As shown in Fig. 1, both OVA peptide as well as OVA IVT RNA pulsed DC were capable of inducing an OVA specific primary CTL response (Fig. 1). RNA pulsed DC were consistently more effective stimulators than peptide pulsed DC. To test whether RNA isolated from E.G7-OVA cells was capable of sensitizing DC to stimulate a primary, OVA-specific, CTL response, total RNA or poly A⁺ RNA was isolated from E.G7-OVA or EL4 cells and incubated with DC. As shown in Figure 1, DC pulsed with either total or poly A⁺ RNA from E.G7-OVA cells but not from EL4 cells, were capable of inducing a strong OVA specific CTL response. Surprisingly, DC pulsed with unfractionated RNA, total or poly A⁺, were as potent inducers of a primary CTL response as DC pulsed with the OVA peptide encoding a defined CTL epitope.
- 20 Stimulation of a CTL response by (total or poly A⁺) EL4 RNA pulsed DC was only marginally above background and statistically not significant (Compare to lysis of EL4 targets by CTL stimulated with OVA peptide or OVA IVT RNA pulsed DC), reflecting the immunodominance of the OVA epitope and the relative weakness of the EL4 encoded antigens.

- As is illustrated by Fig. 2, total, as well as poly A⁺, but not poly A⁻, RNA isolated from E.G7-OVA cells is capable of sensitizing DC to stimulate a primary CTL response. To prove that sensitization of DC is indeed mediated by RNA, poly A⁺ RNA from E.G7-OVA cells was incubated with either an antisense oligonucleotide spanning the sequence encoding the single CTL epitope present in the chicken ovalbumin gene or with a control oligodeoxynucleotide, and then treated with RNase H to

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remove any RNA sequence to which the oligodeoxynucleotide probe has hybridized. As shown in Fig. 2, induction of a primary, OVA-specific CTL response was abolished when the poly A⁺ RNA was incubated with the antisense, but not with the control, oligodeoxynucleotide. Fig. 2 also shows that cells expressing the complete ovalbumin gene, E.G7-OVA cells, and RMA-S cells pulsed with the 8 amino acid long OVA peptide encoding the single dominant CTL epitope are lysed to a similar extent following stimulation with total or poly A⁺ E.G7-OVA RNA pulsed DC. This indicates, therefore, that the majority of epitopes presented by E.G7-OVA RNA pulsed DC correspond to the previously defined single dominant CTL epitope encoded in the chicken ovalbumin gene.

15 Induction of anti-tumor immunity by DC pulsed with tumor RNA.

This example demonstrates that vaccination of mice with OVA RNA pulsed DC provided protection against a challenge with E.G7-OVA tumor cells. Mice were immunized once with 2 x 10⁶ RNA pulsed DC or with 5 x 10⁶ irradiated E.G7-OVA cells. Ten days later, mice were challenged with a tumorigenic dose of E.G7-OVA cells. Appearance and size of the tumor were determined on a regular basis. Fig. 3 shows the size of the tumors at 37 days post-tumor implantation. The average tumor size in mice immunized with irradiated EL4 cells was 25 cm, while the average tumor size in animals immunized with the OVA expressing EL4 cells (E.G7-OVA) was only 7.03 cm. This difference is a reflection of the high immunogenicity of the chicken OVA antigen expressed in EL4 cells and the poor immunogenicity of the parental, EL4, tumor cell line. Vaccination with DC pulsed with RNA (total or poly A⁺ fraction) derived from E.G7-OVA cells was as effective as vaccination with the highly immunogenic E.G7-OVA cells

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(average tumor size 7 cm). Vaccination with DC incubated with total or poly A⁺ RNA derived from EL4 tumor cells had a slight protective effect (average tumor size: 22 cm and 19.5 cm, respectively) which was not statistically significant, consistent with poor to undetectable immunogenicity of EL4-derived antigens. Consistent with the primary CTL induction data (Fig. 1), vaccination of mice with OVA IVT RNA pulsed DC provided the most effective anti-tumor response (average tumor size: 3.9 cm), while vaccination with the control antisense OVA IVT RNA did not elicit a significant protective response.

The potency of DC pulsed with tumor-derived RNA was further evaluated in the B16/F10.9 (H-2^b) melanoma metastasis model. The B16/F10.9 melanoma tumor is poorly immunogenic, expresses low levels of MHC class I molecules, and is highly metastatic in both experimental and spontaneous metastasis assay systems (Porgador et al., 1996, J. Immunology 156: 1772-1780). Porgador et al. have shown that, when vaccinations are carried out after the removal of the primary tumor implant, only irradiated tumor cells transduced with both the IL-2 and the H-2K^b genes, are capable of significantly impacting the metastatic spread of B16/F10.9 tumor cells in the lung (Porgador et al. 1995, Cancer Research 55: 4941-4949). Thus, the B16/F10.9 melanoma model and the experimental design used by Porgador et al. constitutes a stringent and clinically relevant experimental system to assess the efficacy of adjuvant treatments for metastatic cancer.

To demonstrate that immunization with tumor RNA pulsed DC, in accordance with the invention, was capable of causing the regression of preexisting lung metastases, primary tumors were induced by implantation of B16/F10.9 tumor cells in the footpad. When the footpad reached 5.5-7.5 mm in diameter, the tumors were surgically

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removed. Two days later, mice were immunized with irradiated B16/F10.9 cells, irradiated B16/F10.9 cells transduced with the H-2K^b gene (F10.9K1), or with RNA pulsed DC preparations (Fig. 4). The mice received a total of three vaccinations given at weekly intervals. The average lung weight of a normal mouse is 0.18-0.22 g. Mice treated with PBS (a negative control) were overwhelmed with metastases. The mean lung weight of mice in this treatment group was 0.81 g; approximately three-quarters of the weight was contributed by the metastases, which were too many to count (>100 nodules). A similar metastatic load was seen when mice were treated with irradiated B16/10.9 cells (data not shown), which confirms numerous previous observations that treatment with irradiated B16/F10.9 tumor cells alone has no therapeutic benefit in this tumor model. As also previously shown, immunization with H-2K^b expressing B16/F10.9 cells (F10.9K1, as a positive control) had a modest therapeutic benefit, as indicated by a statistically significant decrease in the average lung weight of the animals in this treatment group. A dramatic response, however, was seen in animals treated with DC that were pulsed with total RNA derived from F10.9 cells in accordance with the invention. The mean lung weight of mice in this treatment group was 0.37 g. A significant dramatic response also was seen in mice treated with DC pulsed with poly A⁺ RNA derived from F10.9 cells in accordance with the invention (average lung weight: 0.42 g). By contrast, no statistically significant decrease in metastatic load was seen in mice treated with DC that were pulsed with either the poly A⁻ RNA fraction derived from F10.9 cells or with total RNA isolated from EL4 tumor cells.

The observation that cells expressing the OVA protein (E.G7-OVA) or cells pulsed with the OVA peptide

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were efficiently lysed by CTL, and the sensitization of DC fractionated with poly A⁺ RNA, strongly suggest that RNA-mediated stimulation of CTL occurs via translation of the input RNA and generation of the predicted class I restricted epitopes, in this case a single dominant epitope encoded in the chicken OVA peptide. These data show that RNA mediated sensitization of DC is more effective than pulsing with peptide because the transfected RNA can serve as a continuous source for the production of antigenic peptides.

Therapeutic Use

The invention can be used to treat or prevent tumor formation in a patient (e.g., melanoma tumors, bladder tumors, breast cancer tumors, colon cancer tumors, prostate cancer tumors, and ovarian cancer tumors). Similarly, the invention can be used to treat or prevent infection in a patient with a pathogen such as a bacterium (e.g., *Salmonella*, *Shigella*, or *Enterobacter*) or a virus (e.g., a human immunodeficiency virus, a Herpes virus, an influenza virus, a poliomyelitis virus, a measles virus, a mumps virus, or a rubella virus).

In treating or preventing tumor formation or pathogen infection in a patient, it is not required that the cell(s) that is administered to the patient be derived from that patient. Thus, the antigen-presenting cell can be obtained from a matched donor, or from a culture of cells grown *in vitro*. Methods for matching haplotypes are known in the art. Similarly, it is not required that the RNA be derived from the patient to be treated. RNA from a donor can be used.

It is preferable that treatment begin before or at the onset of tumor formation or infection, and continue until the cancer or infection is ameliorated. However, as the examples described herein illustrate, the

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- invention is suitable for use even after a tumor has formed, as the invention can cause a regression of the tumor. In treating a patient with a cell or vaccine produced according to the invention, the optimal dosage of the vaccine or cells depends on factors such as the weight of the mammal, the severity of the cancer or infection, and the strength of the CTL epitope. Generally, a dosage of 10^5 to 10^8 RNA-loaded antigen-presenting cells/kg body weight, preferably 10^6 to 10^7 cells/kg body weight, should be administered in a pharmaceutically acceptable excipient to the patient. The cells can be administered by using infusion techniques that are commonly used in cancer therapy (see, e.g., Rosenberg et al., New Eng. J. of Med. 319:1676, 1988). The optimal dosage and treatment regime for a particular patient can readily be determined by one skilled in the art of medicine by monitoring the patient for signs of disease and adjusting the treatment accordingly.
- Where the antigen-presenting cell is used to induce a CTL response *in vitro*, the resulting effector CTLs can subsequently be administered to a mammal in a CTL-based method of therapy (see, e.g., PCT/US91/06441). CTL produced *in vitro* with the antigen-presenting cells of the invention can be administered in a pharmaceutically acceptable excipient to a mammal by employing conventional infusion methods (see, e.g., Rosenberg et al., *supra*). Typically, 10^9 - 10^{10} cells are administered over the course of 30 minutes, with treatment repeated as necessary. Such a CTL-based method of therapy may be combined with other methods, such as direct administration of the antigen-presenting cells of the invention. The CTL and antigen-presenting cells may be autologous or heterologous to the patient undergoing therapy. If desired, the treatment may also include

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administration of mitogens (e.g., phyto-hemagglutinin) or lymphokines (e.g., IL-2 or IL-4) to enhance CTL proliferation.

What is claimed is:

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1. A method for producing an RNA-loaded antigen presenting cell (APC) that presents on its surface a tumor antigenic epitope encoded by tumor-derived RNA, wherein the epitope induces T cell proliferation, said
5 method comprising:

introducing into an antigen-presenting cell *in vitro* tumor-derived RNA comprising tumor-specific RNA that encodes an antigen that induces T cell proliferation and tumor immunity, thereby producing an RNA-loaded APC
10 that presents on its surface a tumor antigenic epitope encoded by the tumor-derived RNA, wherein the epitope induces T cell proliferation.

2. The method of claim 1, wherein said APC is a dendritic cell.

15 3. The method of claim 1, wherein said APC is a macrophage.

4. The method of claim 1, wherein said APC is an endothelial cell.

5. The method of claim 1, wherein said APC is an
20 artificially generated APC.

6. The method of claim 1, wherein said RNA is tumor-derived RNA that comprises poly A⁺ RNA.

7. The method of claim 1, wherein said RNA is tumor-derived RNA that comprises cytoplasmic RNA.

25 8. The method of claim 1, wherein the RNA is introduced into the APC by contacting the APC with the RNA in the presence of a cationic lipid.

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9. The method of claim 1, wherein said RNA is tumor-derived RNA that is provided as a fractionated tumor extract that is fractionated with respect to a non-RNA component of the tumor extract.

5 10. The method of claim 1, further comprising introducing into the APC RNA encoding an immunomodulator.

11. The method of claim 10, wherein the immunomodulator is a cytokine.

10 12. The method of claim 10, wherein the immunomodulator is a costimulatory factor.

13. The RNA-loaded APC produced by the method of claim 1.

14. A method for treating or preventing tumor formation in a patient, said method comprising
15 administering to the patient a therapeutically effective amount of the RNA-loaded APC of claim 13.

15. The method of claim 14, wherein the tumor-derived RNA is derived from said patient.

20 16. The method of claim 1, wherein the tumor-derived RNA is derived from fixed tissue.

17. The method of claim 14, wherein the tumor-derived RNA is derived from a donor patient.

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18. A method for producing a cytotoxic T lymphocyte that is cytotoxic for a cell which presents a tumor antigen (CTL), said method comprising:

providing a T lymphocyte;

5 contacting said T lymphocyte *in vitro* with the RNA-loaded APC of claim 13; and

maintaining said T lymphocyte under conditions conducive to CTL proliferation, thereby producing a CTL that is cytotoxic for a cell which presents a tumor
10 antigen.

19. A CTL produced by the method of claim 18.

20. A method for treating or preventing tumor formation in a patient, said method comprising administering to the patient a therapeutically effective
15 amount of the CTL of claim 19.

21. The method of claim 20, wherein the T lymphocyte is derived from said patient.

22. The method of claim 20, wherein the T lymphocyte is derived from a donor patient.

20 23. The method of claim 20, wherein the tumor-derived RNA is derived from a tumor of said patient.

24. The method of claim 20, wherein the tumor-derived RNA is derived from a donor patient.

25 25. The method of claim 1, wherein the tumor-derived RNA is derived from a melanoma.

26. The method of claim 1, wherein the tumor-derived RNA is derived from a bladder tumor.

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27. The method of claim 1, wherein the tumor-derived RNA is derived from a tumor selected from the group consisting of breast cancer tumors, colon cancer tumors, prostate cancer tumors, and ovarian cancer tumors.

28. The method of claim 1, wherein said RNA is isolated from a cell.

29. The method of claim 1, wherein said RNA is prepared by amplification and *in vitro* transcription.

30. The method of claim 1, wherein said RNA is tumor-derived RNA that comprises nuclear RNA.

31. The method of claim 1 wherein said RNA comprises a minigene.

32. The method of claim 1, wherein said RNA is prepared by *in vitro* transcription.

33. A method for producing an RNA-loaded antigen presenting cell (APC) that presents on its surface a pathogen antigenic epitope encoded by the RNA, wherein the epitope induces T cell proliferation, said method comprising:

introducing into an antigen-presenting cell *in vitro* pathogen-derived RNA consisting essentially of RNA encoding a pathogen antigen that induces T cell proliferation and an immune response to the pathogen, thereby producing an RNA-loaded APC that presents on its surface a pathogen antigenic epitope encoded by the RNA, wherein the epitope induces T cell proliferation.

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34. The method of claim 33, wherein said APC is selected from the group consisting of dendritic cells, macrophages, and endothelial cells.

35. The method of claim 33, wherein said APC is an artificially generated APC.

36. The method of claim 33, wherein said RNA is tumor-derived RNA that comprises poly A⁺ RNA.

37. The method of claim 33, wherein said pathogen-derived RNA is derived from a virus.

38. The method of claim 37, wherein said virus is selected from the group consisting of Hepatitis viruses, human immunodeficiency viruses, influenza viruses, poliomyelitis viruses, measles viruses, herpes viruses, mumps viruses, and rubella viruses.

39. The method of claim 33, wherein said pathogen-derived RNA is derived from a bacterium.

40. The method of claim 39, wherein said bacterium is selected from the group consisting of *Salmonella*, *Shigella*, and *Enterobacter*.

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41. A method for producing a cytotoxic T lymphocyte (CTL) that is cytotoxic for a cell which presents a pathogen antigen, said method comprising:

providing a T lymphocyte;

5 contacting said T lymphocyte *in vitro* with the RNA-loaded APC of claim 33; and

maintaining said T lymphocyte under conditions conducive to CTL proliferation, thereby producing a CTL that is cytotoxic for a cell which presents a tumor

10 antigen.

42. A CTL produced by the method of claim 41.

43. A method for treating or preventing pathogen infection in a patient, said method comprising

administering to the patient a therapeutically effective
15 amount of the CTL of claim 42, wherein said APC is loaded with pathogen-derived RNA.

44. The method of claim 18, wherein the tumor-derived RNA comprises at least 80% of polyA⁺ RNA naturally present in a tumor cell.

20 45. The method of claim 44, further comprising detecting sensitization of the contacted T lymphocyte as an indication of the induction of a CTL response.

46. The method of claim 45, wherein sensitization is detected in a cytotoxicity assay that comprises
25 detecting killing of an RNA-loaded cell that presents on its surface a tumor or pathogen antigenic epitope encoded by RNA.

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47. The method of claim 45, wherein sensitization of the contacted T lymphocyte is detected as an increase in cytokine secretion by the T lymphocyte.

48. The method of claim 1, wherein said RNA comprises a sequence that encodes a polypeptide which controls intracellular trafficking of a polypeptide to which it is attached ("trafficking sequence").

49. The method of claim 1, wherein said trafficking sequence is KDEL (SEQ ID NO: 1); KFERQ (SEQ ID NO: 2); QREK (SEQ ID NO: 3); MAISGVPVLGFFIIAVLMSAQESWA (SEQ ID NO: 4); a pentapeptide comprising Q flanked on one side by four residues selected from the group consisting of K, R, D, E, F, I, V, and L; or a signal peptide.

50. The method of claim 33, wherein said RNA comprises a sequence that encodes a trafficking sequence.

51. A method for detecting an increase in tumor-specific or pathogen-specific CTL in a patient, the method comprising:

i) contacting a first sample of T lymphocytes from the patient *in vitro* with RNA-loaded APCs that present a cell-surface tumor or pathogen antigenic epitope encoded by the RNA, thereby producing a first expanded sample of T lymphocytes;

ii) administering to the patient a therapeutically effective amount of the RNA-loaded APCs that present a cell-surface tumor or pathogen antigenic epitope encoded by RNA;

iii) subsequent to the administering step, contacting a second sample of T lymphocytes from the patient *in vitro* with RNA-loaded APCs that present a

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cell-surface tumor or pathogen antigenic epitope encoded by the RNA, thereby producing a second expanded sample of T lymphocytes;

iv) comparing sensitization of the first expanded sample of T lymphocytes with sensitization of the second expanded sample of T lymphocytes, wherein an increased level of sensitization in the second sample, as compared with the first sample is an indicator of an increase in tumor-specific or pathogen-specific CTL.

10 52. The method of claim 51, wherein sensitization is measured in a cytotoxicity assay.

53. The method of claim 1, wherein the tumor-derived RNA is derived from frozen tissue.

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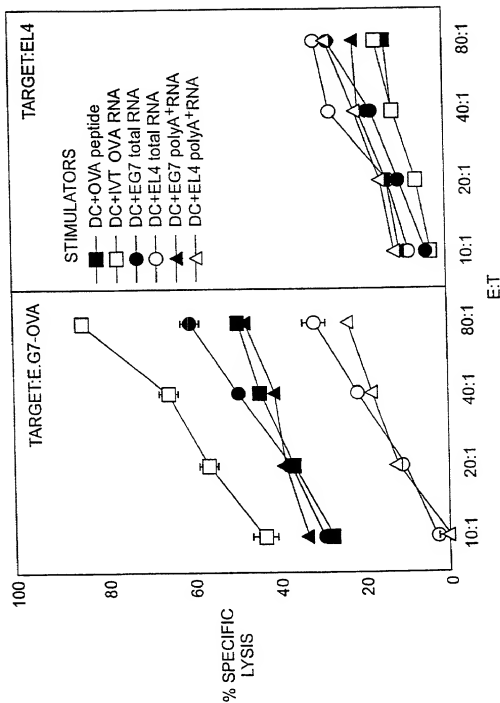


FIG. 1

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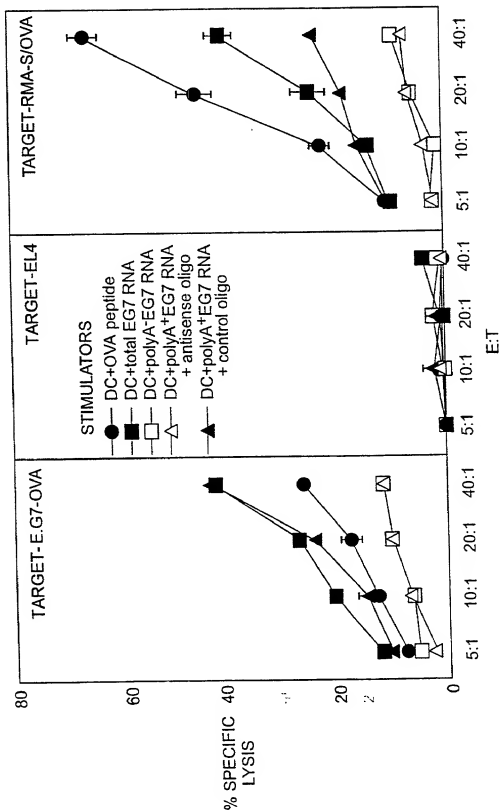


FIG. 2

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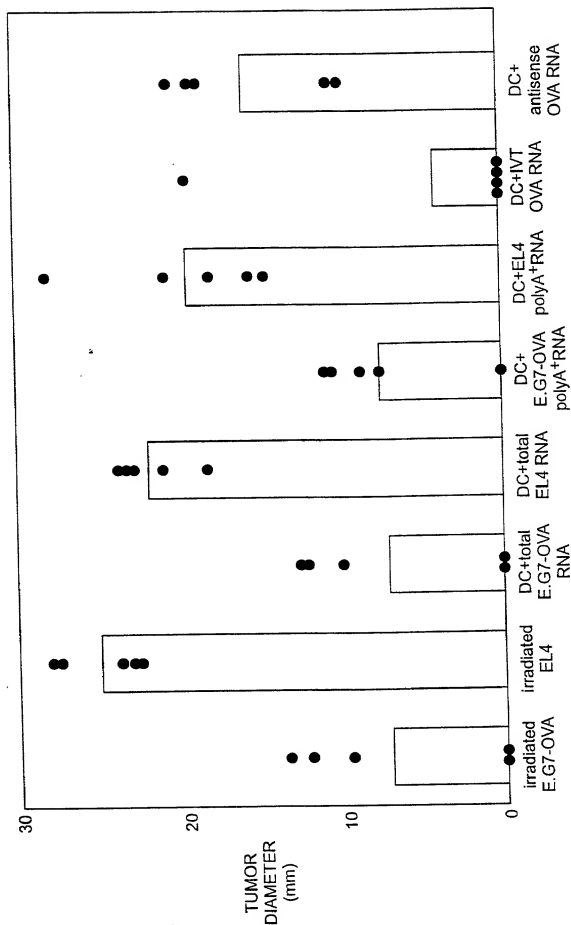


FIG. 3

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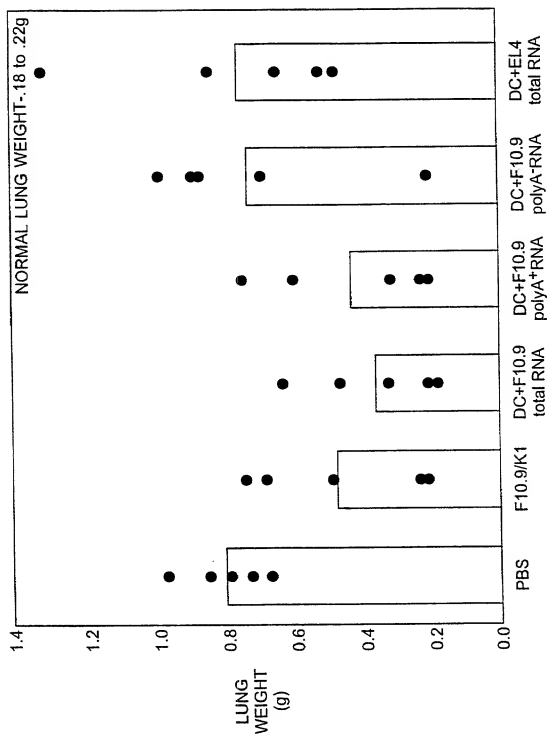


FIG. 4

RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHODS FOR TREATING CANCERS AND PATHOGEN INFECTIONS USING ANTIGEN-PRESENTING CELLS LOADED WITH RNA

the specification of which (check applicable box(es)):

- ☐ is attached hereto
☒ was filed on October 29, 1998 as U.S. Application Serial No. 09/171,916 (Atty Dkt. No. 1579-312)
☒ was filed as PCT international application No. PCT/US97/07317 on 30 April 1997
 and (if applicable to U.S. or PCT application) was amended on _____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:

Priority Foreign Application(s):

Application Number	Country	Day/Month/Year Filed
		30 April 1996

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

Application Number	Date/Month/Year Filed

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56 which occurred between the filing date of the prior applications and the national or PCT international filing date of this application:

Prior U.S./PCT Application(s):

Application Serial No.	Day/Month/Year Filed	Status: patented
PCT/US97/07317	30 April 1997	pending, abandoned

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And I hereby appoint NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed), and the following attorneys thereof (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, 25327; Larry S. Nixon, 25640; Robert A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Farris, 33152; Richard G. Besha, 22270; Mark E. Nusbauer, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchell, 29009; Duane M. Byers, 33363; Jeffrey H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnham, Jr. 29366; Thomas E. Byrne, 32205; Mary J. Wilson, 32955; J. Scott Davidson, 33488; Alan M. Kagen, 36178; William J. Griffin, 31260; Robert A. Molan, 29834; B. J. Sadoff, 36663; James D. Berquist, 34776; Updeep S. Gill, 37334; Michael J. Shea, 34725; Donald L. Jackson, 41090.

1. Inventor's Signature: Smita Nair Date: 1/26/99
 Inventor: Smita (first) K (last) Nair Indian
 Residence: (city) Durham (state/country) North Carolina
 Post Office Address: 4710 Buttonbush Drive, Durham, North Carolina
 (Zip Code) 27712
2. Inventor's Signature: David Boczkowski Date: 1-26-99
 Inventor: David (first) J (last) Boczkowski U.S.A.
 Residence: (city) Durham (state/country) North Carolina
 Post Office Address: 4710 Buttonbush Drive, Durham, North Carolina
 (Zip Code) 27712
3. Inventor's Signature: Gilboa Date: 1/26/99
 Inventor: Gil (first) MI (last) Gilboa U.S.A.
 Residence: (city) Durham (state/country) North Carolina
 Post Office Address: 3604 Stonegate Drive, Durham, North Carolina
 (Zip Code) 27705

FOR ADDITIONAL INVENTORS, check box ☐ and attach sheet with same information and signature and date for each.